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# Dose–Response and Age- and Temperature-Related Susceptibility of the Diamondback Moth (Lepidoptera: Plutellidae) to Two Isolates of *Beauveria bassiana* (Hyphomycetes: Moniliaceae)

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**ABSTRACT** Two isolates of *Beauveria bassiana* (Balsamo) Vuillemin were used in bioassays against the diamondback moth, *Plutella xylostella* (L.). One isolate, ARSEF 4543, originated from a diamondback moth larva collected in New York and the other, Mycotech GHA, is the active ingredient in Mycotrol, a product registered for control of insect species belonging to several orders. Dose–response assays of suspended spores sprayed on larvae resulted in similar $LD_{50}$s (518 and 614 spores per square centimeter for ARSEF 4543 and Mycotech GHA, respectively) but different slopes for the probit regression lines (0.64 and 0.89). Survival times for larvae inoculated at a range of dosages were variable for ARSEF 4543 but decreased with increasing dosages for Mycotech GHA. Third and 4th instars were more susceptible than 2nd instars, although survival times varied between the 2 isolates for different instars. Larvae inoculated early in the 2nd or 3rd instar had shorter suvival times than those inoculated later in each instar. Spores of *B. bassiana* were readily detected on exuviae from inoculated larvae using fluorescence microscopy, indicating the significance of molting as a means of avoiding infection. The isolates did not differ significantly in their activities over a range of temperatures. Highest mortality and lowest survival times were observed at 25°C; mortality decreased and survival time increased at temperatures both above and below 25°C. These findings provide baseline information on the susceptiblity of *P. xylostella* to *B. bassiana* and they indicate the potential utility of a commercial preparation of *B. bassiana* for use against *P. xylostella* in the field.

**KEY WORDS** *Plutella xylostella*, *Beauveria bassiana*, microbial control, bioassay, entomopathogenic fungus, virulence

THE DIAMONDBACK MOTH, *Plutella xylostella* (L.), is the most important pest worldwide of cultivated brassicas (Talekar and Shelton 1993). Chemical insecticides are used extensively against this pest, but it has developed resistance to many, including some formulations of *Bacillus thuringiensis* Berliner (Shelton et al. 1993).

Several fungi have been isolated from *P. xylostella* (Humber 1992), but few have been studied in detail. Recent descriptions of natural epizootics of 2 Entomophthorales species in Asian populations of *P. xylostella* (Riethmacher and Kranz 1994) have led to more detailed work on *Zoophthora radicans* (Brefeld) Batko infection of *P. xylostella* by others (Pell et al. 1993a, b). In Malaysia, Ibrahim and Low (1993) have shown the potential effectiveness of *Beauveria bassiana* (Balsamo) Vuillemin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith against *P. xylostella* in the field. More recently, Shelton et al. (1998) and Vandenberg et al. (1998) have shown that commercial formulations of *B. bassiana* can control *P. xylostella* populations on crucifer seedlings in screenhouses and on larger plants in the field.

As a component of a research program to explore the use of *B. bassiana* for control or management of pest insects, we have developed a laboratory assay protocol to test the efficacy of *B. bassiana* and other fungi against *P. xylostella*. Elsewhere we report screening results against *P. xylostella* for 55 isolates of *B. bassiana*, *Fusarium* sp., *Metarhizium anisopliae* (Metschnikoff) Sorokin, and *Paecilomyces farinosus* (Holm ex Gray) Brown & Smith (Vandenberg and Ramos 1997). The objectives of this study were to determine the dose response and age- and temperature-related susceptibility of *P. xylostella* larvae to 2 *B. bassiana* isolates. One of these was isolated from *P. xylostella* and the other was isolated from a commercial *B. bassiana* product.

## Materials and Methods

The *B. bassiana* isolate ARSEF 4543 was obtained from the USDA–ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY). This is a single-spore isolate derived from a single infected *P. xylostella* larva collected in Ontario County, NY. A 2nd isolate, Mycotech GHA, was obtained from the tech-

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nical powder of Mycotrol (Mycotech, Butte, MT), a *B. bassiana* product registered for control of certain species of Orthoptera, Homoptera, Heteroptera, Thysanoptera, and Coleoptera. Each isolate was initially passed through *P. xylostella* larvae and each was subcultured <5 times before use in assays. Cultures were maintained on Sabouraud dextrose agar plus 2% yeast extract (SDAY) at 24°C and a photoperiod of 15:9 (L:D) h. Fungal conidia were scraped from the surface of cultures 14–21 d old and suspended in 0.01% Tween 80 by vortexing for 2 min. Suspensions were filtered through 16 layers of sterile cheesecloth to remove clumps and hyphal fragments. Spore concentrations were estimated using a hemacytometer and adjusted as needed, using additional 0.01% Tween 80, for experiments described below. *P. xylostella* larvae treated only with the suspending medium (0.01% Tween 80) were included in each assay as controls. (Mortality among control larvae was minimal and no evidence of *B. bassiana* infection was detected.)

First instars of *P. xylostella* were obtained from A. M. Shelton (Geneva, NY) and incubated on wheat germ diet (Shelton et al. 1991) at 10°C and a photoperiod of 15:9 (L:D) h for 1–2 d. Larvae were used as 2nd instars except as described below. For assays, larvae were transferred to petri dishes, 7–10 per dish, lined with moistened filter paper. Four dishes were assigned randomly to each treatment for each experiment. Insects were inoculated at room temperature (≈22°C) using a Burgerjon spray tower (Burgerjon 1956) equipped with a Spraying Systems 2850 nozzle (Wheaton, IL). The nozzle was calibrated according to Vandenberg (1996) by spraying spore suspensions at a range of concentrations onto water agar and estimating their concentration by counting the spores. A suspension volume of 5 ml was applied to each group of 4 dishes of larvae at an air flow rate of 5 liters/min and at a dosage described below. After inoculation, two 1-cm disks of *P. xylostella* diet (Bioserv Premix) were added to each dish. The dishes were sealed with parafilm and incubated in darkness at 25°C (except as described below). After 24 h, any dead insects were discarded and not counted. Living larvae were transferred to a sterile petri dish with fresh diet and incubated at 25°C (except as described below) and a photoperiod of 15:9 (L:D) h. Larvae were monitored daily and diet was replaced as needed for 7 d. Dead larvae were removed to petri dishes containing a piece of moistened filter paper. Death from fungal infection was confirmed by observing characteristic fungal eruption from cadavers and subsequent sporulation. To confirm viability of spore suspensions, 5 ml of each was sprayed on SDAY plates. Plates were incubated at 25°C and a photoperiod of 15:9 (L:D) h for 2–3 d until identifiable fungal colonies appeared. Colony counts confirmed deposition at the rates indicated (data not shown).

**Dose–Response Assays.** Larvae were inoculated with each isolate at dosages of 312, 625, 1,250, 2,500 and 5,000 spores per square centimeter. Assays were repeated 4 times for ARSEF 4543 and 3 times for Mycotech GHA. Probit analysis (SYSTAT 1997) was used to estimate $LD_{50}$s, fiducial limits, and other regression parameters. Analysis of variance (ANOVA) was used to test the effect of dosage on survival time in days. Means were compared using the Tukey test (SYSTAT 1996).

**Age-Related Susceptibility.** First instars were incubated at 25°C and a photoperiod of 15:9 (L:D) h until they reached the 2nd, 3rd, or 4th instar. Newly molted larvae of each instar with visibly darkened cuticles were chosen for the tests. Seven to eleven larvae of each instar were placed in each of 4 dishes. Larvae were inoculated with either ARSEF 4543 or Mycotech GHA at a dosage of 625 spores per square centimeter and observed daily for 7 d. The entire experiment was repeated twice. Analysis of variance was used to test for the effects of isolate and instar on angular-transformed percentage mortality and on survival time in days. Means were compared using the Tukey test (SYSTAT 1996).

To determine the effect of age within and instar, groups of 200 second and 3rd instars were subdivided into groups of 100. Half were inoculated on the day of selection and the other half were inoculated the following day (20–24 h later). For inoculation, 2 ml of a suspension of Mycotech GHA at $2 \times 10^7$ spores per milliliter was added to 100 mm sterile petri dishes lined with filter paper. Larvae were placed in the dishes without food for 1 h and then transferred individually to unlined petri dishes and provided with a small cube of *P. xylostella* diet. Dishes were sealed with parafilm. Larvae were checked twice daily for molting and mortality. Cast exuvia were recorded and removed when found, and the instar at death was recorded. Dead larvae were incubated for diagnosis as described above. Time to molt, survival time, and instar at death were compared for early- versus late-instar larvae using Student *t*-tests or the Pearson $\chi^2$ frequency distribution test (SYSTAT 1996).

For microscopy, additional larvae were inoculated as described above. Upon molting, cast skins were mounted in a drop of 0.01% Calcofluor white M2R (Sigma, St. Louis, MO) on a microscope slide with glass coverslip. Cuticle from the same recently molted larvae was removed by dissection and mounted in Calcofluor. Observations were made using an Olympus BH2 microscope with a mercury-lamp epifluorescence illuminator with an excitation wavelength of 390–440 nm (violet), chromatic beam splitter at 460 nm, and barrier filter at 475 nm.

**Temperature-Related Susceptibility.** Second instars were inoculated at a dosage of 625 spores per square centimeter. After inoculation, larvae were incubated in darkness at 15, 20, 25, 30, or 35°C for 24 h and then transferred to new dishes, as described above, and maintained at the same assigned temperature. The experiment was repeated 5 times using both fungal isolates. Mortality was assessed after 10 d for the 1st experiment and daily for 10 d for the other 4 experiments. Five larvae were used per dish and there were 4 dishes per treatment. Analysis of variance was done to determine the effects of isolate and temperature on angular-transformed percentage mortality and on survival time in days. Means were compared using the

**Table 1. Probit analyses of 2 isolates of *B. bassiana* against 2nd instars of the diamondback moth**

| Isolate | No. larvae | Slope ± SE | $LD_{50}$ (95% FL) (spores/cm$^2$)[a] | $\chi^2$ |
|---|---|---|---|---|
| ARSEF 4543 | 691 | 0.64 ± 0.12 | 518 (271–763) | 3.9 |
| Mycotech GHA | 423 | 0.89 ± 0.13 | 614 (222–1,930) | 4.3 |

Four assays for ARSEF 4543, 3 for Mycotech GHA, 7–10 insects per replicate, 4 replicates per dose, 5 doses per assay.
[a] Analyses done on $\log_{10}$ spores per square centimeter.

Tukey test (SYSTAT 1996). Nonlinear regression analysis was used to analyze the effect of temperature on survival time in days (SYSTAT 1996).

## Results

**Dose–Response Assays.** Probit analysis was significant for ARSEF 4543 and for Mycotech GHA (Table 1). $LD_{50}$s were similar for the 2 isolates, and fiducial limits overlapped considerably, indicating no significant difference. A significant difference between the slopes was indicated by the interaction between isolate and dosage ($\chi^2 = 0.002$, df = 1, $P < 0.04$). Average survival time varied significantly with dosage for ARSEF 4543 ($F = 7.0$; df = 4, 483; $P < 0.0001$) and for Mycotech GHA ($F = 20.3$; df = 4, 408; $P < 0.0001$) (Table 2). For Mycotech GHA there was a decrease in survival time with increasing dosage. Survival times for ARSEF 4543 were lower at the 2 highest dosages and at an intermediate dosage.

**Age-Related Susceptibility.** The 2 fungal isolates differed significantly in their effect on angular-transformed percentage mortality ($F = 12.6$; df = 2, 42; $P < 0.0001$) and on average survival time ($F = 23.6$; df = 1, 373; $P < 0.0001$) among larvae of different instars, so the effect of age on susceptibility was examined separately for each isolate. Mortality varied significantly among instars for each isolate (for ARSEF 4543: $F = 4.3$; df = 2, 21; $P < 0.03$; for Mycotech GHA: $F = 7.6$; df = 2, 21; $P < 0.003$) (Table 3). Survival times also varied significantly among instars for each isolate (for ARSEF 4543: $F = 5.4$; df = 2, 172; $P < 0.005$; for Mycotech GHA: $F = 11.3$; df = 2, 201; $P < 0.0001$). For ARSEF 4543, significantly higher mortality and survival times were observed for 3rd instars. In contrast, for Mycotech GHA, highest mortality was observed among 4th instars, and lowest survival times were observed for 3rd and 4th instars.

Larvae inoculated earlier within the 2nd or 3rd instar had significantly lower survival times than those inoculated later in the instar (Table 4; for 2nd instars: $t = -1.93$, df = 135, $P < 0.05$; for 3rd instars: $t = -4.77$, df = 66, $P < 0.001$). The age distribution by stage at death did not vary significantly between larvae inoculated early versus late within the 2nd instar (Pearson $\chi^2 = 5.2$, df = 3, $P < 0.16$), but it did vary significantly for larvae inoculated early versus late in the 3rd instar (Pearson $\chi^2 = 29.5$; df = 2; $P < 0.001$). Larvae inoculated early in the 3rd instar were most likely to die as larvae whereas larvae inoculated late in the 3rd instar were most likely to die as pupae.

Microscopic observations confirmed that spores of *B. bassiana* were abundant on exuvia from larvae inoculated late within the 2nd or 3rd instar (i.e., those larvae that molted soon after inoculation). Spores were rarely detected on cuticles dissected from larvae inoculated early in either instar.

**Temperature-Related Susceptibility.** Larval mortality and survival time varied significantly with temperature (for mortality: $F = 7.8$; df = 4, 42; $P < 0.0001$; for survival time: $F = 7.2$; df = 4, 464; $P < 0.0001$) but not with isolate ($F = 1.0$; df = 1, 42; $P < 0.18$), so isolates were combined for further analysis. Highest mortality was observed among the intermediate temperatures of 20, 25, and 30°C (Table 5). Lowest survival times were observed at 25 and 30°C. Mortality data were fit to a nonlinear, quadratic model: percentage mortality =

**Table 2. Survival times (mean ± SEM) for 2nd-instars of the diamondback moth inoculated with *B. bassiana***

| Isolate | Dosage (spores/cm$^2$) | No. mycoses[a] | Survival time, d |
|---|---|---|---|
| ARSEF | 312 | 71 | 5.3 ± 0.2a |
| | 625 | 83 | 4.6 ± 0.1b |
| | 1,250 | 103 | 5.2 ± 0.1a |
| | 2,500 | 118 | 4.8 ± 0.1ab |
| | 5,000 | 113 | 4.5 ± 0.1b |
| Mycotech GHA | 312 | 78 | 4.6 ± 0.1a |
| | 625 | 86 | 4.2 ± 0.1b |
| | 1,250 | 85 | 4.0 ± 0.1bc |
| | 2,500 | 85 | 3.8 ± 0.1cd |
| | 5,000 | 79 | 3.5 ± 0.1d |

Means for each isolate followed by the same letter are not significantly different (Tukey test, $P < 0.05$).
[a] Combined results of 3–4 experiments (see text).

**Table 3. Percentage mortality (mean ± SEM) and survival times for larvae of the diamondback moth inoculated at different ages with *B. bassiana***

| Isolate | Instar | No. larvae[a] | % mortality | No. mycoses | Survival time, d |
|---|---|---|---|---|---|
| ARSEF 4543 | 2 | 53 | 53 ± 11b | 28 | 4.4 ± 0.4a |
| | 3 | 86 | 86 ± 3a | 74 | 5.0 ± 0.2b |
| | 4 | 84 | 72 ± 7b | 60 | 4.6 ± 0.3a |
| Mycotech GHA | 2 | 65 | 74 ± 6b | 48 | 4.5 ± 0.2a |
| | 3 | 87 | 83 ± 8b | 72 | 4.0 ± 0.2b |
| | 4 | 83 | 100 ± 0a | 83 | 4.2 ± 0.1b |

Means for each isolate followed by the same letter are not significantly different (Tukey test, $P < 0.05$).
[a] Combined results of 2 experiments (see text).

**Table 4. Survival times (mean ± SEM) for diamondback moth inoculated as 2nd or 3rd instars with *B. bassiana* isolate Mycotech GHA**

| Stage at inoculation | No. inoculated | No. mycoses | Survival time, h |
|---|---|---|---|
| Early 2nd instar | 100 | 82 | 73 ± 3a |
| Late 2nd instar | 98 | 55 | 82 ± 4b |
| Early 3rd instar | 100 | 58 | 61 ± 4a |
| Late 3rd instar | 98 | 10 | 106 ± 6b |

Means within each instar followed by the same letter are not significantly different (Student $t$-test, $P < 0.05$).

**Table 5. Percentage mortality (mean ± SEM) and survival times of 2nd instars of the diamondback moth inoculated with *B. bassiana* and incubated at different temperatures**

| Temp, °C | No. larvae | % mortality | No. mycoses | Survival time, b[a] |
|---|---|---|---|---|
| 15 | 210 | 18 ± 6b | 28 | 5.8 ± 0.2a |
| 20 | 206 | 52 ± 10a | 105 | 5.9 ± 0.2a |
| 25 | 205 | 72 ± 9a | 132 | 4.9 ± 0.1b |
| 30 | 206 | 56 ± 11a | 70 | 5.3 ± 0.1ab |
| 35 | 208 | 19 ± 6b | 34 | 5.8 ± 0.4a |

Means followed by the same letter are not significantly different (Tukey test, $P < 0.05$).

[a] Combined results for 2 isolates of *B. bassiana*.

$-2.52 + 0.26$ (temperature) $-0.005$ (temperature$^2$). The $r^2$ was 0.40, the residual mean square was 0.07, and the standard errors were 0.54, 0.04, and 0.001 for intercept, temperature, and temperature$^2$, respectively.

## Discussion

The $LD_{50}$s we estimated (5.2 and 6.1 × $10^2$ spores/per square centimeter; Table 1) are lower than some, and higher than other, estimates of *B. bassiana* assayed against lepidopteran larvae. Higher $LD_{50}$s were estimated by Feng et al. (1985) in spray tower assays against *Ostrinia nubilalis* (Hübner). Among their $LD_{50}$ estimates, the lowest was $8.4 \times 10^3$ colony-forming units (CFU)/cm$^2$ for *B. bassiana* isolate ARSEF 533 against the most susceptible instar (1st). Other $LD_{50}$s in their study ranged from $1.3 \times 10^4$ to $5.6 \times 10^7$ CFU per square centimeter. Ignoffo et al. (1982) estimated an $LD_{50}$ of $1.4 \times 10^4$ spores per square centimeter for 1st instars of *Trichoplusia ni* (Hübner) using a leaf-disk treatment. McDowell et al. (1990) also used a leaf-surface treatment in assays against *Elasmopalpus lignosellus* (Zeller). Lower $LD_{50}$s were estimated for 1st instars (12 spores per square centimeter) than for 3rd instars (51 spores per square centimeter).

The slopes of the probit regression lines we estimated (Table 1) are within the range of those commonly estimated in assays of entomopathogens generally (Burges and Thomson 1971), and *B. bassiana* against lepidopteran larvae in particular. Slope estimates for assays of *B. bassiana* were 0.49–1.64 for *O. nubilalis* (Feng et al. 1985), 1.34 *T. ni* (Ignoffo et al. 1982), and 0.50–1.12 for *E. lignosellus* (McDowell et al. 1990).

We observed decreased survival times with increasing dosage for Mycotech GHA, but the trend was less distinct for ARSEF 4543 (Table 2). This may be caused by variability within the isolate, although it originated from a single spore. Perhaps the temperature optimum for ARSEF 4543, used in our study, is lower than the 25°C used in our tests. Carruthers et al. (1985) also observed decreasing survival times with increasing dosage for young larvae of *O. nubilalis*, but the relationship was less significant at higher temperatures and among older larvae. Survival times among 2nd instars reared at 26°C, approximately the same temperature we used, ranged from 1.7 d at their highest dosage ($2.9 \times 10^6$ CFU/cm$^2$) to 3.9 d at their lowest ($2.3 \times 10^4$ CFU/cm$^2$). McDowell et al. (1990) observed decreasing $LT_{50}$s with increasing dosage for both instars tested of *E. lignosellus* (1st and 3rd); times to death were 12–14 d for larvae inoculated at the $LD_{50}$.

We assayed *B. bassiana* against 2nd, 3rd, and 4th instars of *P. xylostella* because these are the feeding stages exposed on host plant leaf surfaces. Second instars were the least susceptible to infection (Table 3). This is caused by the shorter duration of early stages, the consequently increased likelihood of molting soon after inoculation, and subsequent shedding of spores with cuticle among early-stage larvae. Our observations of fungal spores on exuvia from recently inoculated larvae corroborates a similar finding of Vey and Fargues (1977) for larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). The absence of spores on exuvia of larvae inoculated early in the 2nd or 3rd instar indicates that the spores had either been rubbed off through insect movements or had already germinated and penetrated the host cuticle.

In our tests, 4th instars were most susceptible to infection by Mycotech GHA and intermediate in susceptibility to ARSEF 4543. Later instars have longer durations that allow the pathogen more time to establish infection before a molt would remove inoculum (Feng et al. 1985). Although we observed statistically significant differences among survival times for larvae inoculated with either isolate (Table 3), the estimates are all within a range of 1.0 d.

We observed a clear optimum (25°C) among temperatures for both mortality and survival time, but fungus infection occurred at all temperatures (Table 5). In contrast, Carruthers et al. (1985) obtained no infection of *O. nubilalis* larvae at 36°C and variable results at 31°C. They also observed lower survival times at lower temperatures.

*Plutella xylostella* larvae vary in their susceptibility to infection by *B. bassiana* based on dosage, age, and temperature. We have shown that ARSEF 4543, originally isolated from a *P. xylostella* larva, is not significantly more efficacious than the commercial isolate Mycotech GHA. We also have shown that *P. xylostella* is susceptible to *B. bassiana* during all of the exposed feeding stages (2nd–4th instar) and over a range of temperatures common in the field.

Vandenberg and Ramos (1997) found no significant differences in infectivity among several *B. bassiana* isolates originating from *P. xylostella* and few differences among 46 *B. bassiana* isolates. This is not surprising given the extremely broad host range of this fungus (Goettel et al. 1990, Feng et al. 1994). Mycotrol (with Mycotech GHA as the active ingredient) is labelled for use against insects in several orders, but not including Lepidoptera. However, Shelton et al. (1998) found that Mycotrol provided control of *P. xylostella* larvae resistant to *B. thuringiensis* on crucifer seedlings and suggested that *B. bassiana* may be useful as a control agent against insecticide-resistant populations. Vandenberg et al. (1998) found that Mycotrol

applications on cabbage plants at the 10–12 leaf stage provided effective control of *P. xylostella*. However, action thresholds for *P. xylostella* are much lower on mature plants (Anonymous 1998), when damage to developing vegetative and flowering heads has greater impact. Current studies are aimed at evaluating the integration of *B. bassiana* with insecticides and other tools for season-long management of pests of crucifers.

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